REMARKS

Claims 27-36, 39, and 42-45 are currently pending in this application. Claim 43 is withdrawn from consideration as being drawn to a non-elected invention. Claims 44 and 45 are objected to for being in improper form. Claims 27-36, 39, 42, 44, and 45 are rejected under 35 U.S.C. § 112, first paragraph, for lack of written description. Claims 42 is rejected under 35 U.S.C. § 103(a) for obviousness. Claims 27-36, 39, 42-45 are rejected for obviousness-type double patenting over claims 1-11 of U.S. Patent No. 6,372,432. Finally, the specification is objected to for improper incorporation of patent and non-patent documents by reference. By this reply, Applicants cancel claims 28, 34-36, 39, 42-43, and 45, amend claims 27 and 29-33, add new claim 46, and address each of the objections and rejections. Applicants reserve the right to pursue cancelled subject matter in a divisional application.

Support for the Amendments

Support for the amendment to claim 27 is found in the specification at, e.g., page 1, lines 4-9, page 3, lines 1-4 and 24-28, and page 4, line 7, through page 5, line 11. Support for the amendment to claims 33 and 46 is found in the specification at, e.g., page 3, lines 6-22. Claims 29-32 have been amended to place the claims in proper claim format. No new matter is added by the amendment.

Information Disclosure Statement

Applicants note that the Form PTO-1449 that was filed with the Information Disclosure Statement on March 5, 2002 has not been initialed and returned. Applicants respectfully request that the Examiner initial and return the Form PTO-1449.

Claim Objections

The Examiner objects to claims 44 and 45 because, as dependent claims, they are "separated from their respective independent claims by a plurality of other independent claims" (Office Action, p. 2). Applicants have canceled claims 44, and 45. This objection may now be withdrawn.

Objection to the Specification

The Examiner objects to the specification, stating:

> The specification is objected to as documents have been improperly incorporated by reference. It is noted with particularity that the instant disclosure makes reference to various foreign patent document[s], both published and unpublished, as well as non-patent publications which are in turn being relied upon for disclosing how the claimed invention is to be made and used. Office Action, p. 2.

The Examiner further states that the language used in the specification "fails to specify what specific information applicant seeks to incorporate by reference and just where the specific information is to be found in each of the cited documents" (Office Action, p. 3). The Examiner also cites *Advanced Display Systems Inc. v. Kent State University*, 54 USPQ2d at 1679 (Fed. Cir. 2000), which states in relevant part:

> To incorporate material by reference, the host document must identify with detailed particularity what specific material it incorporates and clearly indicate where that material is found in the various documents.

Finally, the Examiner directs Applicants to M.P.E.P. § 608.01(p)(I)(A), which states:

> In addition to other requirements for an application, the referencing application should include an identification of the referenced patent, application, or publication. Particular attention should be directed to specific portions of the referenced document where the subject matter

being incorporated may be found.

Applicants respectfully disagree with the Examiner's objection to the specification. The patent and non-patent documents recited in the specification are merely provided as background references that can be consulted by the skilled artisan.

For example, the technique of subtractive hybridization, which is discussed on page 17, line 25, through page 19, line 7, has been known since at least 1981, as is evidenced by Vitek et al. (Nucl. Acids Res. 9:1191-1202, 1981; a copy of which is provided). As one embodiment of the invention, the specification discloses the subtractive hybridization method reported by Kohne et al. (Biochemistry, 16:5329-5341, 1977) and Miller and Riblet (Nucl. Acids Res. 23:2339-2340, 1995), which is referred to as phenol emulsion reassociation technique (PERT). This subtraction based genomic cloning method is but one possible method that can be used by the skilled artisan, as is made clear by the specification on page 18, lines 26-28, which states: "Any other hybridization method in liquid phase, preferably in emulsion, can be used within the scope of the present invention. Furthermore, the hybridization can also be done with one of the strands immobilized on a support." Thus, practicing the method of present claims 27, 29-33, 44, and 46 does not require performance of the methods of Kohne et al. and Miller and Riblet. Thus, these references have been properly cited in the specification.

The reference in the specification to international patent application PCT/FR 99/00547, noted by the Examiner on page 2 of the Office Action, is also provided as background for the skilled artisan. PCT/FR 99/00547, which was published as WO 99/46403 on September 16, 1999, is cited in the present specification for its disclosure of methods, e.g., the "DATAS methodology," for the preparation of nucleic acid libraries for use in the method of present claims 27, 29-33, 44, and 46. Each of the methods disclosed in PCT/FR 99/00547 are disclosed

in enabling detail in the present specification. For example, the specification teaches the DATAS methodology, stating:

> banks can be prepared by hybridization between the nucleic acid population derived from cells isolated from the blood in a pathological situation, and the nucleic acid population derived from circulating cells in the control situation, and isolation, from the hybrids formed, of the nucleic acids corresponding to differential splicing. (Page 17, lines 18-22.)

The specification also discloses several other methods, all of which are well known in the art, which can be used by the skilled artisan for preparing the nucleic acid molecules of the library, including, e.g., high flow sequencing electronic subtraction, serial analysis of gene expression (SAGE), nucleic acid arrays, differential display, and subtractive cloning (see page 12, line 15, through page 14, line 8). Thus, the techniques and methods disclosed in PCT/FR 99/00547 are adequately disclosed in the present specification, and the skilled artisan can easily practice the method of present claims 27, 29-33, 44, and 46 based on the present specification without the need to consult PCT/FR 99/00547. Thus, reference to PCT/FR 99/00547 in the specification is provided merely as background for the skilled artisan.

If the Examiner disagrees with Applicants' position, Applicants would be willing to provide a Declaration stating that the methods and techniques disclosed by the cited references are either fully enabled by the teachings in the present specification or are so well known in the art that the skilled artisan would need no further disclosure in the specification pertaining to these references for the practice of the method of present claims 27, 29-33, 44, and 46. In any event, Applicants respectfully request that the Examiner's objection to the specification be withdrawn.

Rejections under 35 U.S.C. § 112, first paragraph

Claims 27-36, 39, 42, 44, and 45 are rejected under 35 U.S.C. § 112, first paragraph, for lack of written description. The Examiner states that "[t]he claim(s) contain subject matter which was not described in the specification in such a way as to reasonably convey to one skilled in the relevant art that the inventor(s), at the time the application was filed, had possession of the claimed invention" (Office Action, p. 5). Applicants respectfully disagree, but have cancelled claims 28, 34-36, 39, 42-43, and 45. Thus, the rejection as it applies to these claims can now be withdrawn. Applicants have also amended claims 27 and 29-33, and added new claim 46 to more clearly recite the subject matter Applicants regard as their invention.

As presently amended, independent claim 27 recites an *in vitro* method for detecting the presence of a given, predefined pathological condition in a human subject. The method involves three steps: i) providing a sample of blood cells (comprising lymphocytes, macrophages, monocytes, or dendritic cells) from a subject (i.e., a test subject who is being tested for the presence of the given, predefined pathological condition), ii) preparing nucleic acid molecules from the sample, and iii) obtaining a hybridization profile by hybridizing all or part of the nucleic acid molecules prepared from the sample of blood cells obtained from the subject with at least one nucleic acid library. The nucleic acid library is prepared from differentially spliced ribonucleic acid molecules (RNAs) expressed in blood cells from human subjects having the given, predefined pathological condition, and the RNAs are specific for the pathological condition sought to be detected. Because the nucleic acid molecules of the library are markers for the pathological condition, hybridization between the nucleic acid library and nucleic acid molecules from a test subject indicates the presence of the given, predefined pathological condition in the test subject.

As noted in the previous Reply to Office Action:

> An advantage of Applicants' invention is that neither the sequences of the nucleic acid molecules of the test sample, *nor the sequences of the nucleic acid molecules of the reference sample (i.e., the "library") need to be known to practice the invention*. All that is required is that the skilled artisan be able to obtain the nucleic acid molecules of the test sample and reference samples and to hybridize the two samples. Neither procedure (i.e., the preparation of the nucleic acid molecules or their hybridization) requires anything more than routine skill in the art. (Page 15, Reply to Office Action, August 4, 2004).

This advantage is acknowledged by the Examiner in the present Office Action, which states that "agreement is reached in that one may not need to know the nucleotide sequence of the nucleic acids that comprise the test sample or the reference" (see page 14 of the Office Action). Thus, by the Examiner's own admission, these elements of Applicants' method satisfy the written description requirement. Because each step of Applicants' method can be performed without knowing the sequence of the test nucleic acid molecules or the nucleic molecules of the library, a written description of these sequences, beyond what is already provided by Applicants' specification, is unnecessary.

In addition, the Examiner raises the issue of whether the specification satisfies the written description requirement with respect to "how one [skilled in the art] is to draw any conclusion when the pathological condition is not associated with any gene splicing event in any blood cell, but is the result of a condition found in non-blood cells" and "just what [hybridization] profiles are to be associated with which pathological condition(s) in any and all mammalians" (Office Action, p. 9). Applicants note that it is the direct or indirect contact of blood cells with the diseased cells or tissue in the subject, which may include blood or non-blood cells, that triggers the expression of differentially spliced RNAs characteristic of the pathological condition in the

blood cells (see the specification at, e.g., page 4, line 25, through page 5, line 11). Thus, any pathological condition that promotes the expression of differentially spliced RNAs in a subject's blood cells can be detected using the method of present claims 27, 29-33, 44, and 46, regardless of whether the pathological condition is associated with blood cells or non-blood cells. Furthermore, because the nucleic acid library is prepared from differentially spliced ribonucleic acid molecules (RNAs) expressed in blood cells from human subjects having the given, predefined pathological condition, as is discussed above, any hybridization between nucleic acid molecules of the test sample and the nucleic acid library indicates the presence of the pathological condition in the test subject. Thus, it is unnecessary for Applicants to describe the hybridization profiles because any hybridization indicates a positive result.

Finally, the Examiner states:

> The claimed methods...have been interpreted as encompassing virtually any pathological condition, as well as any degree of exposure to a pathological condition. Said "exposure" has been construed as encompassing, but not limited to, having the cells of an individual, e.g., a health care provider, coming into direct or indirect contact with an individual that has a pathological condition. Said "pathological condition" has also been construed as encompassing diseases of unknown etiology, as well as individual being in the same general environment (e.g., a room) where a pathological agent (virus, bacteria, fungi, amoeba, helminth, carcinogen, etc.) is also to be found wherein [s]aid pathological agent is capable of causing a disease. Said "pathological condition" has also been construed as encompass[ing] exposure to UV light normally associated with sunlight. Office Action, p. 8.

Applicants point out that claim 27, as presently amended, recites that the pathological condition is one that is given and predefined, meaning that the pathological condition sought to be detected in the human subject is one that is known by the skilled artisan. Thus, pending claims 27, 29-33, 44, and 46 no longer encompass diseases of unknown etiology. Furthermore, the method of

claims 27, 29-33, 44, and 46 does not encompass "any degree of exposure to a pathological condition," as indicated by the Examiner. Rather, the method involves the detection of a pathological condition in a subject by determining whether the blood cells of the subject have come into direct or indirect contact with diseased cells or tissues within the body of the subject; the contacting does not occur outside of the body. Thus, Applicants believe that claims 27, 29-33, 44, and 46, as presently amended, address this issue raised by the Examiner.

For all of the reasons provided above, Applicants' specification clearly satisfies the written description requirement of 35 U.S.C. § 112, first paragraph, by providing a thorough description of the claimed invention with all of its limitations such that one skilled in the art can reasonably conclude that Applicants were in possession of the claimed invention (see M.P.E.P. § 2163). Accordingly, Applicants respectfully request that the rejection of claims 27-36, 39, 42, 44, and 45 under 35 U.S.C. § 112, first paragraph, for lack of written description be withdrawn, and that the rejection should not be applied to new claim 46.

Rejections under 35 U.S.C. § 103(a)

Claim 42 is rejected under 35 U.S.C. § 103(a) "as being unpatentable over applicant's representative admissions in the response of 28 October 2004." Office Action, p. 18. The Examiner states:

> Applicant's representative admits at page 17 that: "Applicants' invention is not the discovery of the nucleic acids [sic] molecules specific for the pathological condition per se, rather, it is the discovery of the...[use] of those nucleic acid molecule in detection methods...[and] the genus of nucleic acid molecules recited in present claims27-36, 38, and 42 for use as the 'library' would have been known to the skilled artisan at the time of filing the present application and obtaining these nucleic acid molecules would not have been new or unconventional in the art."
> In view of the foregoing admission by Applicant's representative as to

> what constitute [sic] the invention of applicant, claim 42 is rejected under 35 U.S.C. 103(a)... Office Action, p. 18

Applicants respectfully disagree that the above admission provides the basis for the rejection under 35 U.S.C. § 103(a) of a claim directed to a kit that includes a support having a plurality of nucleic acid molecules deposited thereon, in which the nucleic acid molecules are specific for differentially spliced gene products present in a mammalian blood cell exposed to or experiencing a pathological condition. In the interest of expediting prosecution of present claims 27, 29-33, 44, and 46, Applicants have cancelled claim 42. This rejection can now be withdrawn.

Obviousness-Type Double-Patenting Rejections

Claims 27-36, 39, and 42-45 are rejected under the judicially-created doctrine of obviousness-type double patenting as being unpatentable over claims 1-11 of U.S. Patent No. 6,372,432. Applicants will submit a terminal disclaimer, if necessary, to overcome this rejection once otherwise allowable subject matter has been determined.

CONCLUSION

Applicants submit that the claims are now in condition for allowance, and such action is respectfully requested.

Enclosed is a petition to extend the period for replying for two months, to and including June 21, 2005, and a check for the fee required under 37 C.F.R. § 1.17(a).

If there are any charges or any credits, please apply them to Deposit Account No. 03-2095.

Respectfully submitted,

Date: June 21, 2005

Kristina Bieker-Brady, Ph.D.
Reg. No. 39,109

Clark & Elbing LLP
101 Federal Street
Boston, MA 02110
Telephone: 617-428-0200
Facsimile: 617-428-7045

Volume 9 Number 5 1981 Nucleic Acids Research

The isolation of ecdysterone inducible genes by hybridization subtraction chromatography

Michael P.Vitek*, Susan G.Kreissman† and Robert H.Gross†

*Department of Biochemistry, Dartmouth Medical School, and †Department of Biological Sciences, Dartmouth College, Hanover, NH 03755, USA

Received 27 October 1980

ABSTRACT

We have developed a procedure for selectively enriching a mRNA population for inducible sequences. Other than the induced mRNA species, the population of mRNA in control cells is approximately the same as the mRNA population in induced cells. Cytoplasmic mRNA from control cells is bound to oligo (dT)-cellulose and used as a template for reverse transcriptase, the oligo (dT) serving as a primer. After removing the template mRNAs, the cDNA-cellulose column is used to hybridize a population of mRNAs from induced cells. The non-hybridized poly A^+ RNAs are greatly enriched in the inducible sequences. We have used this technique of hybridization subtraction chromotography to select a mRNA population enriched for the mRNAs inducible by ecdysterone in Schneider's Line 2 Drosophila cells. This population of RNAs was used to screen a recombinant library. Preliminary results indicate that approximately 10% of the RNA in the probe population represents ecdysterone inducible sequences. Methods are described for optimizing the cDNA synthesis reaction (we obtain $\geq$ 30% efficiency) and hybridizing RNA to the cDNA-cellulose resin. This method can be used to select induced mRNAs regardless of the way in which the induction is brought about.

INTRODUCTION

The isolation of a pure gene depends on the availability of a good probe. Gene isolation has been accomplished in a number of ways. The isolation of some mRNAs, for example globin mRNA (1,10,13,14), has been possible because the population of mRNAs in certain specific cell types is greatly enriched in the desired mRNA. Other RNAs, like ribosomal RNA, were isolated by taking advantage of their unusual base composition (3,12,17). Each of these cases has relied on some special property of the RNA to allow its isolation. Either the system was producing large amounts of a product (to make mRNA isolation feasible) or the gene had an unusual base composition.

The isolation of a particular RNA sequence comprising a very small portion of the entire cellular RNA complement has been very difficult. This is a particularly challenging problem when dealing with the phenomenon of gene induction, since the induced product is often only a minor component of

the cell's mRNA population. The technique of hybridization subtraction chromatography, described in this paper, lends itself readily to the isolation of a population of mRNAs greatly enriched in inducible mRNA sequences. In essence, the technique removes mRNAs from an induced cell's mRNA population that are also found in control cell mRNA populations. The remaining mRNAs are greatly enriched for the induced sequences. The method has broad applicability since it can select low abundance mRNA sequences from a highly complex mRNA population. This is true regardless of the mechanism employed to induce these low abundance RNAs. Further, once the cDNA-cellulose column is constructed, it can be used repeatedly without resynthesing more cDNA.

In the present work, we demonstrate the feasibility of hybridization subtraction chromatography by purifying an RNA population enriched for the mRNAs induced in Schneider Line 2 Drosophila cells by treatment with ecdysterone. These induced RNAs represent less than 0.2% of the cytoplasmic mRNA population of ecdysterone treated cells. Using the column purified mRNA as a probe, we have been able to select a number of recombinant clones containing DNA that hybridizes significantly to induced cell mRNAs, but not to control cell in mRNAs.

MATERIALS AND METHODS

CELLS AND LABELING

Schneider's Line 2 (SL2) cells were grown in 75 cm^2 plastic tissue culture flasks to a density of 3.0 to 4.5 X 10^6 cells per ml in Schneider's Drosophila medium (GIBCO) supplemented with 0.1 volumes of heat inactivated fetal calf serum (GIBCO). Hormone treated cultures were exposed to 1.0 µM ecdysterone (Sigma) for 4 hours and then harvested. In some experiments, RNA was labeled in vivo with 2.5 µCi per ml ^{3}H-uridine (Amersham) for 4 hours.

RNA ISOLATION

Cells were lysed by resuspending cell pellets in lysis buffer (0.3 M sucrose, 2 mM $MgAc_2$, 3 mM $CaCl_2$, 10 mM tris (pH 7.4), 0.1% Triton X-100, 5mMDTT) containing 8 mM vanadium-adenosine complex (2), incubating for 10 minutes on ice, and homogenizing with the tight fitting A pestle in a Dounce homogenizer. Cell lysis was monitored by phase contrast microscopy until ≥ 90% of the cells had been broken. Nuclei were pelleted for 5 minutes in an HB-4 swinging bucket rotor at 10,000 RPM (7600xg). RNA from the cytoplasmic supernatant was deproteinized by repeated phenol/chloroform/isoamyl alcohol (50:49:1) extractions at room temperature and precipitated with ethanol overnight at -20°C. Poly A^+ RNA was prepared by oligo (dT)-

cellulose chromatography (P-L Biochemical, type 7 Oligo (dT)-cellulose, ref 19). All poly A^+ RNAs were bound to oliog (dT)- cellulose a minimum of three times before use.

SYNTHESIS OF cDNA-CELLULOSE

In the standard cDNA-cellulose synthesis reaction, 600 µg of 3 times oligo (dT)-cellulose retained cytoplasmic RNA from control cells was bound to 360 mg of oligo (dT)-cellulose in 20 ml of 50 mM Tris-HCl (pH 8.3), 20 mM dithiothreitol, 10 mM $MgCl_2$, 100 mM KCl, 0.6 mM dTTP, 0.4 mM each of dATP, dGTP, dCTP; 4.0 mM sodium pyrophosphate and 1.14% ethanol. Synthesis of cDNA was initiated by addition of 550 units per ml avian myeloblastosis virus reverse transcriptase (generously supplied by Dr. J. Beard, Life Sciences, St. Petersburg, FL) and 20 µCi per ml ^{3}H-dGTP; synthesis was allowed to continue for 60 minutes at 37°C. The reaction was terminated by transferring the resin to a 40°C water jacketed column and washing with hybridization buffer [50% formamide (BRL), 0.1% SDS (BDH), 10 mM Tris-HCl (pH 7.6), 0.6 M NaCl, 10 mM EDTA and 20 µg per ml polyadenylic acid (Sigma)] until the column eluate had an $A_{260} \leq 0.05$. To denature template RNAs from their cDNA complements which are covalently linked to the cellulose matrix [through the oligo (dT) bridge], the temperature was raised to 60°C and the column washed with elution buffer [50% formamide, 0.1% SDS, 10 mM Tris-HCl (pH 7.6)] until the eluate had an $A_{260} \leq 0.05$. RNA collected from both low and high temperature washes were ethanol precipitated. The oligo (dT)-cDNA-cellulose resin was cooled to room temperature, washed and stored in 95% ethanol at -20°C until needed.

HYBRIDIZATION OF RNA TO cDNA-CELLULOSE

(^{3}H)-poly A^+ cytoplasmic RNA from ecdysterone treated cells was hybridized to the cDNA-cellulose (10-fold cDNA excess) in hybridization buffer for 48 hours at 40°C with 60 strokes per minute agitation. The reaction volume was 10 µl per mg of cDNA-cellulose resin. After the hybridization was complete, the entire mixture was poured into a 40°C water jacketed column, allowed to settle for 1 to 2 hours, and then washed with hybridization buffer (at 40°C) to remove the non-hybridized RNAs until the eluate was ≤ 100 CPM per ml above background. Hybridized RNAs were then released from the column by heating to 60°C and washing with 60°C elution buffer. The hybridization/washing procedure was repeated twice more on the "non-bound" RNA fraction from the first hybridization reaction. The RNA that did not bind to the column after three cycles of hybridization was then applied to an oligo (dT)-cellulose column as described above. The poly A^+ RNA obtained from this last column

was used to screen the recombinant library.

SCREENING THE RECOMBINANT LIBRARY

A portion of the putative ecdysterone induced RNA probe was labeled with ^{125}I by a modification of the method of Schrader and O'Malley (11) and used to screen a complete Drosophila genomic recombinant library obtained through the generosity of E.A. Craig (4), Dept. of Physiological Chemistry, University of Wisconsin. This library contains partial Hind III digested Drosophila DNA inserts of 8.8 Kb average size in pBR322. Filters containing plasmid DNAs with Drosophila inserts were prepared according to the method of Grunstein and Hogness (5) and hybridized to the ^{125}I labeled RNA probe. Hybridization was performed (1.5 ml per filter) in 0.02% each of Ficoll, poly vinyl pyrollidone, and bovine serum albumin; 2 µg per ml E. coli RNA, 50% formamide, 1 mg per ml polyadenylic acid and 5XSSC (1 X SSC = 0.15 M NaCl, 0.015 M Na Citrate, pH 7.0) for 22 hours at 37°C. After hybridization, the filters were washed 10 minutes in 6XSSC, three more times in 2XSSC, air dried and exposed to Kodak XR-5 X-ray film for 24 hours at -70°C with Cronex Lightning Plus (DuPont) intensifying screens.

Positive colonies were picked, grown overnight in L broth, cloned and stored in 96 well microtiter plates (Costar). Duplicate filters were made from clones in the microtiter plates and were hybridized to mRNA from either control cells (-) or ecdysterone treated cells (+). The "+/-" screening method permitted us to identify the induced clones as those which hybridized to induced cell mRNAs but not to control cell mRNAs.

RESTRICTION DIGESTS AND GEL ELECTROPHORESIS

Plasmids were purified as described by Guevry et al. (20) and digested with Hind III restriction endonuclease under conditions recommended by the supplier (Bethesda Research Labs). Reactions were phenol extracted, the DNA ethanol precipitated and analyzed by agarose gel electrophoresis. The gel was 1% agarose in 18 mM NaCl, 20 mM NaAc, 2 mM EDTA, and 50 mM Tris·HCl (pH 8.0). Electrophoresis was carried out at 8V/cm until the bromphenol blue dye front reached the bottom of the gel.

RESULTS AND DISCUSSION

PREPARATION OF cDNA-CELLULOSE

Reverse transcriptase can be used to synthesize cDNA from an RNA template by extending from a base paired primer. In solution, typical reverse transcriptase reactions using oliog (dT) primers hydrogen bonded to poly A tails of

mRNAs synthesize 10-15 µg of cDNA for every 100 µg of mRNA in the reaction (16)- i.e., a 10-15% efficiency. Using a column bound primer often results in even lower efficiencies (15,21). Because of this potential problem, we optimized our reaction in a number of parameters.

Originally, following the conditions of Hirsch, et al. (6,7), we had little success. Switching from potassium acetate and magnesium acetate to KCl and $MgCl_2$ made a significant difference as shown in Table 1. Maximal cDNA synthesis occurs at 100 mM KCl which is the salt concentration used in all subsequent reactions.

The concentration of reverse transcriptase was the next factor optimized. As shown in Table 1, the enzyme optimum is 550 units of reverse transcriptase per ml of reaction mix. The plateau probably results from the saturation of all available elongation sites for the enzyme (i.e. - there are no more oligo (dT) primers hydrogen bonded to mRNAs which can serve as enzyme recognition sites).

After optimal salt and enzyme concentrations were determined, other conditions of the reaction were investigated to improve further the efficiency of our system. Actinomycin D was included in early reactions to minimize the synthesis of double stranded cDNA (9,18). However, we found that actinomycin D significantly reduced the efficiency of our reactions (perhaps by blocking the synthesis of double stranded cDNA) as shown in Table 1. An alternative is the use of sodium pyrophosphate (9) in the reactions to prevent the reverse transcriptase from recopying the cDNA. The inclusion of 4.0 mM sodium pyrophosphate provided high efficiencies while preventing synthesis of double stranded cDNA. With higher levels of synthesis, it seemed logical to increase the triphosphate and Mg concentrations (11). Increasing the dTTP concentration from 0.4 mM to 0.6 mM; dATP, and dCTP from 0.14 mM to 0.4 mM; and $MgCl_2$ from 6 mM to 10 mM increased the efficiency further. As shown in Table 1, the presence of some ethanol in the reaction was necessary but no significant dependence on its concentration was observed. Doubling the amount of oligo (dT)-cellulose from 0.6 mg per µg mRNA to 1.2 mg per µg mRNA results in half the amount of cDNA synthesis per mg oligo (dT)-cellulose. Doubling the amount of template mRNA in the reaction doubles the amount of cDNA synthesized. For any particular oligo (dT)-cellulose resin, there was as much as a 10-fold increase in efficiency when the resin was thoroughly washed with water. This treatment apparently removed any trace contaminants which severely inhibit reverse transcription. Also, oligo (dT)-cellulose from different suppliers or different lots from

TABLE 1

EFFICIENCY OF cDNA SYNTHESIS

KCl	$MgCl_2$	dTTP	dATP dCTP dGTP	Reverse Transcriptase	Actinomycin D	$NaPP_i$	ethanol	Temp.	Relative Efficiency
100 mM KAc	6 mM $MgAc_2$	400 µM	140 µM	55 µ/nk	36 µg/ml	0 mM	1%	42°C	0.43%
100 mM KAc	6 mM	400	140	55	36	0	1	42	0.95
100	6	400	140	55	36	0	1	42	1.10
100	6	400	140	55	0	0	1	42	1.23
100	6	400	140	55	36	0	1	42	1.10
100	6	400	140	55	72	0	1	42	0.92
100	6	400	140	55	36	0	0	42	0.75
100	6	400	140	55	36	0	1	42	1.14
100	6	400	140	55	36	0	2	42	1.29
100	10	400	400	550	36	0	1	42	2.77
100	10	400	400	550	36	0	1	37	8.85
100	6	600	140	0	36	0	1	37	0.0006
100	6	600	140	55	36	0	1	37	0.57
100	6	600	140	550	36	0	1	37	1.52
100	6	600	140	1100	36	0	1	37	1.29
100	6	600	140	1650	36	0	1	37	1.48
100	6	600	140	2200	36	0	1	37	1.02
0	6	600	140	550	36	0	1	37	3.78
50	6	600	140	550	36	0	1	37	5.81
100	6	600	140	550	36	0	1	37	7.14
150	6	600	140	550	36	0	1	37	5.99
200	6	600	140	550	36	0	1	37	4.89
100	10	600	400	550	0	4 mM	1	37	31.3

Assays for incorporation of ^{3}H-dGTP into cDNA were carried out by pelleting the resin after the 60' synthesis reaction, washing two times in hybridization buffer, resuspending in water, and counting the resuspended resin in a triton/toluene fluor.

the same supplier could vary by as much as two orders of magnitude in efficiency of the cDNA synthesis reaction. Lowering the incubation temperature from 42°C to 37°C also increased efficiency. These careful optimization procedures allowed us to increase our efficiency from an original 0.43% to greater than 30%.

To determine what fraction of the template RNA was actually copied into cDNA during the reaction, the oligo (dT)-cDNA-cellulose resin was washed with hybridization buffer at 40°C immediately after the synthesis reaction. This low temperature washing removes RNAs that are bound only by the A:T base pairs (between the poly A mRNA tail and the oligo (dT) of the resin), but will not release most mRNAs that are hydrogen bonded to their cDNAs. The column was then heated to 60°C and washed with elution buffer to denature the template RNAs from their cDNAs which were covalently attached to the cellulose through the oligo (dT) linker. The results showed that 322 µg (54%) of the template RNA was eluted in the low temperature wash while 278 µg (46%) of the template RNA eluted in the high temperature wash. In this reaction, the 278 µg of template RNA were copied to produce 168 µg of cDNA (as determined by ^{3}H-dGTP incorporation). The average length of the cDNA can therefore be estimated to represent ∿60% of the template RNA length. Since extension of the cDNA starts at the 3' end of the mRNA, the cDNA sequences attached to the column are likely to be enriched for sequences complementary to the 3' mRNA ends in comparison to the 5' mRNA end sequences.

RNA ENRICHED FOR ECDYSTERONE INDUCIBLE SEQUENCES

Hybridization of mRNA from ecdysterone treated cells (16 µg) was performed under conditions of cDNA excess (168 µg of cDNA attached to cellulose). After hybridization, the column was regenerated and the RNA not hybridizing the first time was re-incubated with the cDNA-cellulose. This procedure was repeated once more to yield an RNA population that had not hybridized to cDNA-cellulose after three incubations under hybridization conditions. This RNA was then applied to an oligo (dT)-cellulose column to remove RNA fragments (which may have been produced during the various manipulations) that did not contain poly A tails; these RNAs mostly represent sequences from the 5' ends of the RNAs. The non-hybridizing poly A^{+} RNA was enriched in ecdysterone inducible sequences. In typical purifications, about 1.5% of the RNA remains in the last fraction which represents a 67-fold enrichment, although this value was ranged from 0.6% to 7.0%. The RNA purified in this manner was subsequently used as a probe to screen the recombinant DNA library.

ISOLATION OF RECOMBINANT CLONES COMPLIMENTARY TO ECDYSTERONE INDUCED RNAs

The probe obtained by hybridization subtraction chromatography was iodinated *in vitro* with ^{125}I and used to screen a recombinant library. Due to the small amounts of probe available, the library was screened in several steps. Twelve microtiter plates (96 wells in each) were set up with each well containing 22 individually picked recombinants. These plates were grown to saturation and then stored at -20°C after addition of an equal volume of 80% glycerol to each well. Using a homemade transfer device, recombinants from each microtiter plate were transferred in an ordered array to agar/ampicillin petri dishes and grown overnight. Nitrocellulose filters made by the method of Grunstein and Hogness (5) were hybridized to ^{125}I probe RNA. Approximately 25,000 recombinants were screened, corresponding to 1.2 times the Drosophila haploid genome length. After autoradiography, 35 positive colonies were evident, representing 3% of the microtiter wells screened. These first generation positives were picked, grown overnight, appropriately diluted and spread on 10 cm agar/ampicillin to a density of 200 to 250 colonies per dish. Again, Grunstein and Hogness screening was employed to select individual positive clones. Second generation positive clones were picked and grown to saturation in microtiter wells for further examination. A third and final screen determined which of the probe selected clones actually represented ecdysterone inducible sequences. The "+/-" method was employed on duplicate filters prepared from microtiter plate clones. One filter was hybridized to mRNA from control cells (-), and the other to mRNA from ecdysterone treated cells (+). Positives were scored if they hybridized to a significantly greater extent with induced cell mRNA than with control cell mRNA. The results of our preliminary "+/-" screen are shown in Figure 1.

Of the 192 second generation positives, 20 were shown to be inducible sequences by the "+/-" procedure. These 20 third generation positives correspond to 10 of the 35 first generation positive wells detected in the initial screen. Therefore, we estimate that 20/192 or 10.4% of the probe prepared by hybridization subtraction chromatography represented ecdysterone inducible sequences.

Seven of these clones were then chosen at random and analyzed by digestion with Hind III and agarose gel electrophoresis. As shown in figure 2, there are six distinctly different patterns of DNA fragments for the seven clones, which implies that these clones originated from different regions of the genome. Thus, our procedure has given us a set of clones,

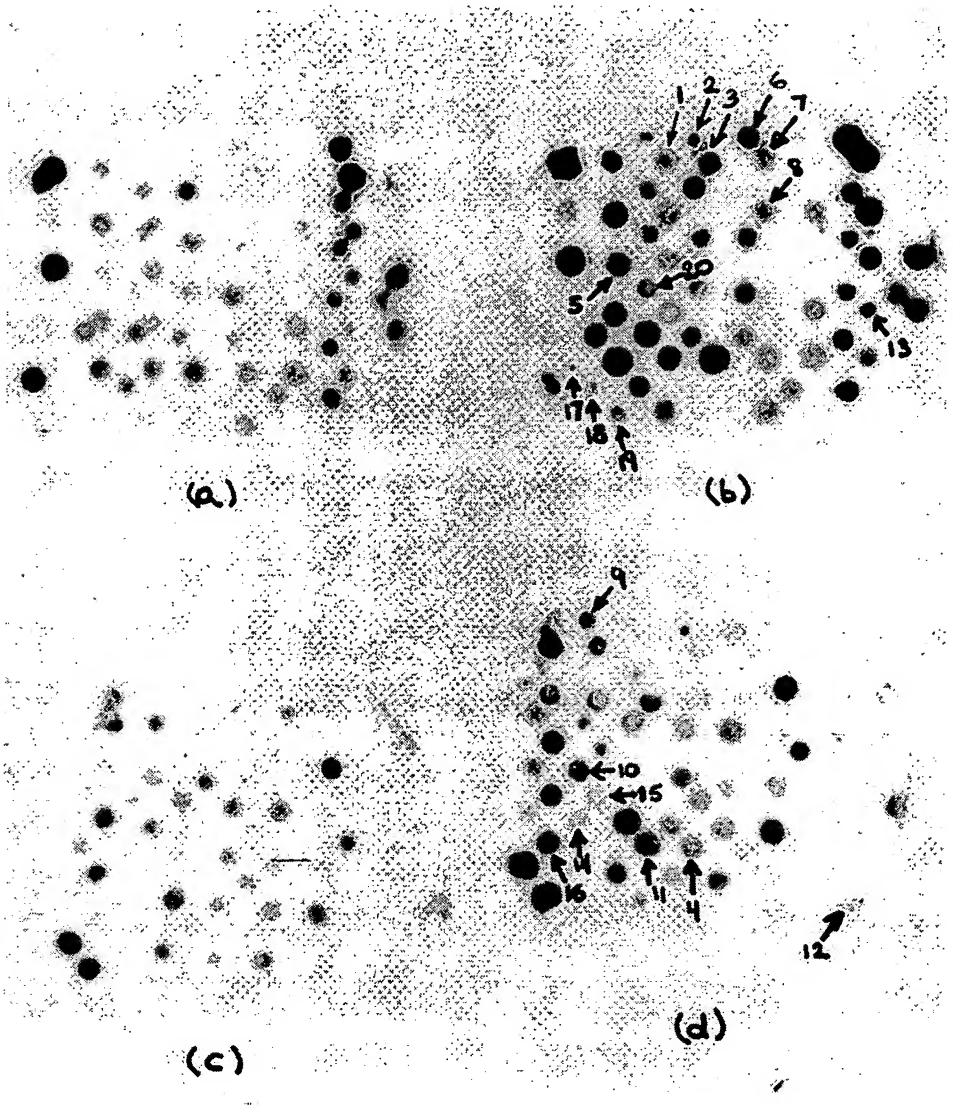

Figure 1. +/- Screening of Candidate Clones. A total of 192 candidate clones were screened (96 in each of two microtiter plates - a/b and c/d) by hybridization with mRNA from ecdysterone treated cells (b and d) or mRNA from control cells (a and c). The clones in (b) are the same as those in (a), and the clones in (d) are the same as those in (c). Colonies 1-4 hybridize with induced cell mRNA but show no detectable hybridization with control cell mRNA. Colonies 5-20 show quantitative differences only, in that they hybridize very weakly with control cell mRNA but strongly with induced cell mRNA.

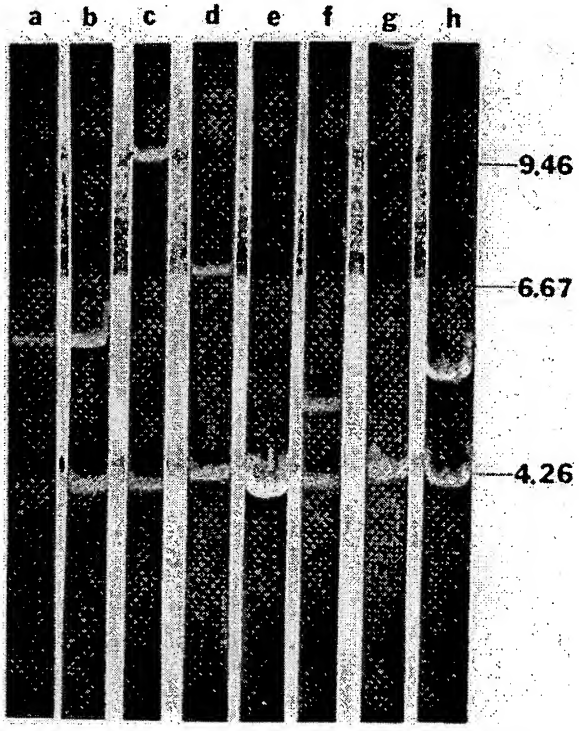

Figure 2. Electrophoretic Analysis of Hind III Digests. Samples were prepared and analyzed as described in materials and methods. Lanes a-d, f-h are recombinant plasmids selected as described in the text. Lane e is pBR322. Size markers are Hind III fragments of λ DNA (size in kilobase pairs).

each containing a sequence(s) inducible by ecdysterone in Schneider line 2 cells. Although our information on the clones is still preliminary, it seems clear that this methodology has been successful.

The cDNA-cellulose resin, once prepared, is quite stable and may be used many times. The resin, containing cDNAs to control cell mRNAs, can be used to purify any inducible sequence regardless of how the mRNA is induced. If some genes expressed in control cells are inhibited by hormone treatment, isolation of those genes may be accomplished by synthesizing cDNA-cellulose using induced mRNAs as template and hybridizing control mRNAs to that cDNA-cellulose. We hope to use our cDNA-cellulose resin to isolate juvenile hormone induced genes, another hormone involved in insect development (8).

Unlike previous techniques, induced mRNAs do not have to be present in

high abundance to be purified. We estimate that the ecdysteroid inducible mRNAs represent less than 0.2% of cytoplasmic mRNAs (1.5% X 10.44% = 0.16%). The enriched mRNA population obtained is approximately 10% ecdysterone inducible sequences. In addition, the technique allows for the isolation of a set of genes, all of which are subject to coordinate induction. The availability of a number of coordinately regulated genes should facilitate the elucidation of molecular control events.

ACKNOWLEDGEMENTS

We thank Dr. Joseph Beard, The Office of Program Resources and Logistics, Viral Cancer Program, National Cancer Institute, Bethesda, MD, for the gift of reverse transcriptase. This research was supported in part by a grant to RHG from the Norris Cotton Cancer Center, Hanover, N.H.

REFERENCES

1. Aviv, H. and Leder, P. (1972) Proc. Natl. Acad. Sci. USA 69:1408-1442.
2. Berger, S. and Birkenmeier, C. (1979) Biochemistry 18:5143-5149.
3. Brown, D.D., Wensink, P.C. and Jordan, E. (1971) Proc. Natl. Acad. Sci. USA 68:3175-3179.
4. Craig, E.A., McCarthy, B.J. and Wadsowrth, S.C. (1979) Cell 16:575-578.
5. Grunstein, M. and Hogness, D. (1975) Proc. Natl. Acad. Sci. USA 72: 3962-3965.
6. Hirsch, F.W., Nall, K.N., Spohn, W.H. and Busch, H. (1978) Proc. Natl. Acad. Sci. USA 75:1736-1739.
7. Hirsch, F.W., Rao, M.R.S., Nall, K.N., Raju, K.S., Shoss, S.M., Spohn, W.H. and Busch, H. (1977) Proc. Am. Assoc. Cancer Res. 18:278.
8. Logan, W.R., Fristrom, D. and Fristrom, J.W. (1975) J. Insect Physiol. 21:1343-1354.
9. Myers, J.C. and Speigelman, S. (1978) Proc. Natl. Acad. Sci. USA 75: 5329-5333.
10. Pevrin, F., Cochet, M., Gerlinger, P., Cami, B., LePennec, J.P. and Chambon, P. (1979) Nuc. Acids Res. 6:2731-2748.
11. Schrader, W.T. and O'Malley, B.W., eds. (1978) Laboratory Methods Manual for Hormone Action and Molecular Endocrinology, pp. 34-40, Houston, TX.
12. Suzuki, Y. and Brown, D.D. (1972) J. Mol. Biol. 63:409-429.
13. Tsai, M.J., Schwartz, R.J., Tsai, S.Y. and O'Malley, B.W. (1975) J. Biol. Chem. 250:5165-5174.
14. Tsai, M.J., Tsai, S.Y. and O'Malley, B.W. (1979) Science 204:314-316.
15. Venetianer, P. and Leder, P. (1974) Proc. Natl. Acad. Sci. USA 71: 3892-3895.
16. Verma, I.M., Temple, G.F., Fan, H. and Baltimore, D. (1972) Nature New Biol. 235:163-167.
17. Wellauer, P.K. and Dawid, I.B. (1977) Cell 10:193-212.
18. Kacian, D.L. and Myers, J.C. (1976) Proc. Natl. Acad. Sci. (USA) 73:3408-3412.
19. Gross, R.H. and Ringler, J. (1979) Biochem. 18(22):4923-4927.

20. Guevry, P., LeBlanc, D.J. and Falkow, S. (1973) J. Bacteriol. 116: 1064-1066.
21. Wood, T.G. and Lingrel, J.B. (1977) J. Biol. Chem. 252:457-463.